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Methods for Inactivation of Venezuelan Equine Encephalitis Virus

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14. ABSTRACT:
We used Venezuelan equine encephalitis (VEE) virus strain TC-83 as a model to study the effects of chemical, thermal, and irradiation conditions on infectivity of single-stranded positive RNA viruses. Our data showed that exposure of the virus to 65 °C for 5–15 min resulted in a 5–6 log reduction of virus infectivity. Treatment with 100–500 mM NaOH caused at least a 4 log reduction in infectivity. Treatment with β-propiolactone at 50–200 mM concentrations for 15–60 min caused complete or near-complete loss of virus infectivity. Treatment with TRIzol LS reagent at a 3:1 (v/v) TRIzol reagent/virus suspension (1×10^7 $TCID_{50}$/mL, where $TCID_{50}$ is the tissue-culture infective dose that will result in 50% culture infection) induced complete loss of infectivity. Effects of increasing doses of cobalt-60 γ irradiation (1–40 kGy) showed a linear inverse relationship between irradiation dose and virus infectivity as measured by $TCID_{50}$ assay. Irradiation doses of >20 kGy caused >4 log reduction in infectivity. At the 40 kGy dose, complete loss of infectivity occurred when a virus titer was 1×10^7 $TCID_{50}$/mL. From these results, we estimate the 1 log reduction dose and sterility assurance level to be approximately 5.5 and 32.9 kGy, respectively.

15. SUBJECT TERMS

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Tissue culture infective dose (TCID)	Irradiation

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PREFACE

The work described in this report was authorized under project no. R.0026476.61.1. The work was started in January 2018 and completed in December 2018. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (CCDC CBC) was known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

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This report has been approved for public release.

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CONTENTS

FIGURES

TABLES

METHODS FOR INACTIVATION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS

1. INTRODUCTION

Single-stranded, positive-sense RNA (SS+RNA) genomes of certain viruses are regulated nucleic acids, given that the intact genomes are capable of producing infectious virus (42 CFR 73.3). Members of this virus class, particularly those in families Flaviviridae and Togaviridae, are known to cause serious encephalitic and hemorrhagic fever illnesses. These viruses are considered potential biological weapons (Croddy et al., 2002).

The Venezuelan equine encephalitis (VEE) virus (genus *alphavirus*, family Togaviridae) is considered a high-priority biothreat agent because of its highly infectious and pathogenic properties as well as the lack of licensed vaccines and drugs for its prevention and treatment (Collins and Kennedy, 1999; Fleming and Hunt, 2006; Steele et al., 2007). This virus comprises six closely related serotypes (I–VI). Its genome consists of approximately 11,700 nucleotides and contains the characteristic features of messenger RNA, namely, a 5′ methylated cap and a 3′ polyadenylated tract; therefore, the intact RNA genome can be used to generate fully functional viral particles.

Several chemical and physical methods for the inactivation of viruses, including SS+RNA viruses, have been used for various clinical, public health, and research applications. Published chemical inactivation methods include treatment of viruses with diethylpyrocarbonate (Stauffer et al., 2007), formalin (Cole et al., 1974), glutaraldehyde (Collins and Kennedy, 1999), alkylating compounds like 1,5-iodonaphthylazide and sodium hydroxide (NaOH) (Lemire et al., 2016; Sharma et al., 2007, 2011), solvent/detergent compounds like tri-*n*-butyl phosphate with Triton X-100 (Roberts, 2008; Hellstern and Solheim, 2011), phenolic and quaternary ammonium compounds (Rutala and Weber, 2008), or RNA-cleaving compounds like RNase (Goncharova et al., 2011).

Virus inactivation by γ irradiation has been studied extensively for health care, food, and public safety applications. Virus inactivation by γ irradiation usually results from the rupturing of covalent bonds in proteins and nucleic acids or the formation of reactive free radicals that interfere with normal protein and nucleic acid functions. The extent of protein and nucleic acid damage is a function of the irradiation dose. Reitman and Tribble (1967) and Gruber (1971) reported that exposure of VEE virus (Trinidad donkey strain) to 60 kGy of γ radiation resulted in the loss of lethality in mice and guinea pigs, as well as the loss of the capacity to produce cytopathic effects (CPEs) in tissue culture.

Here, we report our findings on the effects of TRIzol LS reagent, NaOH, β-propiolactone (BPL), temperature, and γ irradiation on the infectivity of the SS+RNA virus, VEE.

2. MATERIALS AND METHODS

2.1 Cell and Virus Cultures

Vero E6 cells (CRL-1586; ATCC; Manassas, VA) were maintained in accordance with the supplier's recommendations at 37 °C with 5% CO_2 in complete medium (Eagle's minimum essential medium [EMEM]). The EMEM contained 10% fetal bovine serum (FBS) and 1% pen/strep (100 U/mL of penicillin and 100 µg/mL of streptomycin). The cells were split at a 1:4 ratio when they reached approximately 90% confluence.

The VEE TC-83 virus stock (1.6×10^8 $TCID_{50}$/mL of NR-63, derived from ATCC VR-1249, where $TCID_{50}$ is the tissue-culture infective dose that will result in the infection of 50% of cells) was obtained from the Biodefense and Emerging Infections Research Resources Repository at the National Institute of Allergy and Infectious Diseases (Bethesda, MD). Confluent Vero E6 cells, grown over 4 days, were rinsed twice with 1× Dulbecco's phosphate-buffered saline (DPBS) and then infected with the virus at 0.1–1.0 multiplicity of infection in 10 mL of infection media (EMEM with 2% FBS and 1% pen/strep) in T-150 flasks. The infected cells were incubated at 37 °C with 5% CO_2, and gentle agitation was applied every 15 min for the first hour of incubation to allow for virus attachment. After the 1 h incubation, 7 mL of complete medium was added, and the infected cells were placed in the incubator at 37 °C with 5% CO_2. The flasks were checked daily for CPE over 3–4 days, using an inverted phase-contrast microscope. When CPE reached ≥80%, the cell suspensions were harvested (using a sterile cell scraper), collected into sterile 50 mL conical tubes, and centrifuged at 4000×*g* for 15 min at 4 °C. The resultant pellets (which contained virus) were each suspended in 500 µL of sterile DPBS, then pooled, homogenized, divided into 200 µL aliquots, and stored at –80 °C. The supernatant (which also contained virus) was concentrated using Amicon Ultra-15 30 kDa centrifugal filter units (Millipore Sigma; Burlington, MA) in accordance with the manufacturer's protocol. The concentrate retained in the Amicon filter cups was collected, pooled, mixed by vortexing, and then divided into 500 µL aliquots for storage at –80 °C. A fraction of the filtrate from this process was saved to check for traces of virus.

2.2 Determining Viral Titer

The VEE virus titer was determined by adapting the $TCID_{50}$ assay that was originally developed by Reed and Muench (1938). Briefly, 3–4 days before the assay, Vero E6 cells were seeded in 48-well tissue-culture plates at a density of ~5×10^4 cells/well in 500 µL of complete medium. On the day of infection, serial 10-fold dilutions of the viral sample were made in 1 mL of DPBS each (10^{-1} to 10^{-8} or more if necessary). The spent medium from the plated cells was removed, cells were rinsed with DPBS, and 100 µL of each viral dilution was added to each of the six vertical wells. The virus was allowed to adsorb to the cells in a 37 °C environment with 5% CO_2 for 1 h, and gentle agitation was applied every 15 min. After this incubation period was complete, 400 µL of the infection medium was added to each well, and the plates were placed in the incubator (37 °C with 5% CO_2) for 4 days. The virus-exposed cells were monitored daily for the appearance of CPE. The numbers of CPE-positive and -negative wells in each of the dilution replicates were recorded, and the $TCID_{50}$ values were calculated using the Reed–Muench method (Reed and Muench, 1938).

2.3 Heat Treatment

A working virus stock titer of 1.6×10^7 $TCID_{50}$/mL was prepared in sterile DPBS, and 300 µL was dispensed into each of 36 sterile (RNase-free) microcentrifuge tubes. The tubes were incubated at ambient temperature (positive control), and 65, 85**,** and 95 °C for 5, 10, 15, 30, or 60 min. At the end of the incubation period at each temperature condition, the tubes were centrifuged briefly and serially diluted through 10^{-8} in infection medium. The $TCID_{50}$ assay was performed as described in Section 2.2.

2.4 NaOH Treatment

A working virus stock titer of 1×10^8 $TCID_{50}$/mL was prepared in sterile DPBS, and 100 µL was dispensed into sterile RNase-free microcentrifuge tubes. An equal volume of prepared NaOH solution was added to obtain a 100, 300, or 500 mM final concentration, and the tubes were incubated for 15, 30, or 60 min at room temperature. After the incubation was complete, each treated virus sample was suspended in a final volume of 15 mL of sterile DPBS, processed with Amicon Ultra-15 30 kDa centrifugal filter units (in accordance with the manufacturer's instructions) to remove any residual NaOH, and subjected to the $TCID_{50}$ assay as described in Section 2.2.

2.5 BPL Treatment

A working virus stock titer of 1×10^8 $TCID_{50}$/mL was prepared in sterile DPBS, and 100 µL of the stock was dispensed into sterile RNase-free microcentrifuge tubes. An equal volume of prepared BPL was added to obtain a final concentration of 50, 100, or 200 mM, and the tubes were incubated at 15, 30, or 60 min at room temperature. After the incubation was complete, each treated virus sample was suspended in a final volume of 15 mL of sterile DPBS, processed with Amicon Ultra-15 30 kDa centrifugal filter units (in accordance with the manufacturer's instructions) to remove BPL, and subjected to the $TCID_{50}$ assay as described in Section 2.2.

2.6 TRIzol Treatment

A working VEE virus stock titer of 1×10^7 $TCID_{50}$/mL was prepared in sterile DPBS. From this, 250 µL of virus stock was mixed with 750 µL of TRIzol LS reagent (Invitrogen; Waltham, MA) in sterile RNase-free microcentrifuge tubes and incubated for 15 min at ambient temperature. Samples were processed either by dialysis with Slide-A-Lyzer 20 kDa cutoff cassettes (Thermo Fisher Scientific; Waltham, MA) or using Amicon centrifugal filters. During the dialysis, two 1000-fold exchanges of sterile DPBS were performed at 4 °C overnight. For centrifugal filtration, the samples were diluted according to the manufacturer's recommendations to remove the TRIzol reagent. The material recovered after dialysis and filtration was assessed for virus infectivity using the $TCID_{50}$ assay.

2.7 γ-Irradiation Exposure

Virus titers ranging from 8×10^6 to 1×10^7 $TCID_{50}$/mL were used for one preliminary study and two confirmatory studies. The virus was exposed to increasing doses of irradiation (1, 5, 10, 15, 20, and 40 kGy). Three test samples were used for each irradiation dose, and 3–6 positive controls (exposed to no irradiation) of the same virus titer were used as a

reference to calculate the log reduction that resulted from exposure to irradiation. Samples were kept on dry ice during the irradiation and stored at –80 °C until they were used for the $TCID_{50}$ assays. Irradiation was carried out using a cobalt-60 source, and doses were measured using an alanine-based dosimeter (Bruker; Billerica, MA).

3. RESULTS AND DISCUSSION

3.1 Heat Inactivation

A preliminary experiment was carried out to determine the appropriate range of temperatures and times that should be examined. In this initial experiment, 100 µL of the working virus stock (1.6×10^7 $TCID_{50}$/mL) was tested at ambient temperature (the positive control) and at 65, 85, and 95 °C for 15, 30, and 60 min. The $TCID_{50}$ assays showed that temperatures of ≥65 °C resulted in complete loss of virus infectivity. These results are consistent with a previous study by Lelie et al. (1987), where Sindbis virus, which is closely related to VEE virus, was completely inactivated by heating at 65 °C for 15 min.

In an attempt to validate these results, another experiment was performed to test the effect of 65 °C temperature on the virus infectivity at 15, 30, and 60 min. Three replicates were conducted for each condition, and the means were used to calculate the log_{10} $TCID_{50}$ and the log reduction. Again, there was complete loss of viral infectivity with exposure to 65 °C for 15, 30, or 60 min (data not shown).

Given that 65 °C at ≥15 min resulted in complete loss of infectivity, another experiment was performed to test the effect of the same temperature (65 °C) at 15 min or less. Table 1 shows that there was virtually complete loss of infectivity at 5, 10, and 15 min, albeit there were a few random occurrences of CPE that were estimated to be <2 viral particles.

Table 1. Effect of 65 °C Following 5, 10, and 15 min Incubation Times on VEE TC-83 Virus Infectivity

Log Dilution	$TCID_{50}$ Value						
	RT (Positive Control)			65 °C			Negative Control
	5 min	10 min	15 min	5 min	10 min	15 min	
–1	6.0	6.0	6.0	0.3	1.3	1.0	0.0
–2	6.0	6.0	6.0	0.0	0.0	0.7	0.0
–3	6.0	6.0	6.0	0.0	0.0	0.0	0.0
–4	6.0	6.0	6.0	0.0	0.0	0.0	0.0
–5	6.0	6.0	6.0	0.0	0.0	0.0	0.0
–6	2.0	5.0	3.0	0.0	0.0	0.0	0.0
–7	0.0	0.3	0.0	0.0	0.0	0.0	0.0
–8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Log $TCID_{50}$	5.75	6.43	6.00	0.03	0.14	0.17	0.00
$TCID_{50}$	5.6E+05	2.7E+06	1.0E+06	1.1E+00	1.4E+00	1.5E+00	1.0E+00
Log reduction	NA	NA	NA	5.72	6.29	5.83	NA

3.2 NaOH Inactivation

Table 2 shows the results of NaOH treatment followed by filtration with Amicon Ultra-15 30 kDa MW cutoff, as described in Section 2.1. The results indicate that the highest log reduction in virus infectivity (5.6 log) was achieved at treatment with 500 mM NaOH for 60 min. However, slightly lower log-reduction values ranging from 4.4 to 5.2 log were achieved with NaOH 100, 300, or 500 mM treatments for 15, 30, or 60 min. These results suggest that NaOH can inactivate the VEE TC-83 virus within a range of 100–500 mM concentrations; however, we recommend treatment with 500 mM for 60 min. Because heat treatment at 65 °C for 5–60 min resulted in complete loss of infectivity (Table 1), we expect that a combination of NaOH (500 mM) and heat (65 °C) treatment for 60 min should result in at least a 6 log reduction in infectivity.

Table 2. Effect of NaOH on VEE TC-83 Virus Infectivity

Log Dilution	$TCID_{50}$ Value										
	100 mM			300 mM			500 mM			Positive Control	Negative Control
	15 min	30 min	60 min	15 min	30 min	60 min	15 min	30 min	60 min		
–1	6.0	6.0	5.0	6.0	5.0	3.0	6.0	5.0	1.0	6.0	0.0
–2	1.0	2.0	2.0	0.0	2.0	0.0	1.0	0.0	0.0	6.0	0.0
–3	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	6.0	0.0
–4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.0	0.0
–5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.0	0.0
–6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0
–7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
–8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0
Log $TCID_{50}$	1.6	1.8	1.5	1.6	1.6	1.0	1.6	1.4	0.6	6.2	0.0
$TCID_{50}$	4E+01	6E+01	3E+01	4E+01	4E+01	1E+01	4E+01	3E+01	4E+00	2E+06	0E+00
Log reduction	4.6	4.4	4.7	4.6	4.6	5.2	4.6	4.8	5.6	NA	NA

3.3 BPL Inactivation

Table 3 shows the results of BPL treatment followed by filtration with Amicon Ultra-15 30 kDa MW cutoff, as described in Section 2.1. The results indicate that the highest log reduction in virus infectivity (6.3 log) was achieved at 50 mM BPL treatment for 15 min. Log reductions between 4.7 and 5.8 were achieved with all three concentrations examined (50, 100, and 200 mM). However, there were unexpected results with 100 and 200 mM treatments for 30–60 min, where a log reduction of approximately 2 was achieved. These results may be due to contamination of the assay cells with residual amounts of the cytotoxic BPL.

Table 3. Effect of BPL on VEE TC-83 Virus Infectivity

Log Dilution	$TCID_{50}$ Value										
	50 mM			100 mM			200 mM			Positive Control	Negative Control
	15 min	30 min	60 min	15 min	30 min	60 min	15 min	30 min	60 min		
–1	0.0	1.0	1.0	1.7	1.5	6.0	4.3	2.0	6.0	6.0	0.0
–2	0.0	1.0	1.0	1.7	2.0	5.0	0.0	1.0	5.0	6.0	0.0
–3	0.0	0.0	1.0	0.0	1.0	4.0	0.0	1.0	5.0	6.0	0.0
–4	0.0	0.0	1.0	0.0	2.0	4.0	0.0	1.0	5.0	6.0	0.0
–5	0.0	0.0	0.0	0.0	1.0	5.0	0.0	0.0	5.0	5.7	0.0
–6	0.0	1.0	0.0	0.0	0.0	4.0	0.0	0.0	4.0	3.0	0.0
–7	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	2.0	0.0	0.0
–8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Log $TCID_{50}$	0.0	0.6	0.9	0.5	1.59	4.3	1.3	1.19	4.4	6.26	0.0
$TCID_{50}$	1E+00	4E+00	8E+00	3E+00	4E+01	2E+04	2E+01	2E+01	3E+04	2E+06	0E+00
Log reduction	6.26	5.66	5.4	5.8	4.7	2.0	5.0	5.1	1.9	NA	NA

3.4 TRIzol Inactivation

TRIzol inactivation of viruses has been described in several publications; however, an accurate quantitative assessment of virus-induced CPE without the interference of TRIzol reagent-induced cytotoxicity has only recently been published (Kochel et al., 2017; Kumar et al., 2015; Haddock et al., 2016). In this study, we used two methods to mitigate the CPE of TRIzol reagent, namely, dialysis and centrifugal filtration. Table 4 shows the results of TRIzol treatment followed by dialysis with 20 kDa MW cutoff, as described in Section 2.1. The results indicate that TRIzol treatment with a 3:1 (v:v) TRIzol reagent/virus sample ratio for 15 min caused complete loss of infectivity, which equates to an approximately 5.2 log reduction. Similarly, when centrifugal filtration was used, there was complete loss of infectivity (data not shown). It is noteworthy that use of either dialysis or centrifugal filtration caused reduction of $TCID_{50}$ by about 0.6 to 1.3 log. The results of TRIzol inactivation were expected and are consistent with those from several other published works that were obtained using various virus species.

Table 4. Effect of TRIzol Reagent on VEE TC-83 Virus Infectivity
(Dialysis Method of TRIzol Removal)

Log_{10} Dilution	Test Samples Mean	Positive Control		Negative Control
		Mean	SD	
–1	0	6	0	0
–2	0	6	0	0
–3	0	6	0	0
–4	0	6	0	0
–5	0	3.3	1.2	0
–6	0	0	0	0
–7	0	0.3	0.6	0
–8	0	0	0	0
Log_{10} $TCID_{50}$	0	5.2		0
$TCID_{50}$	0	1.4E+05		0
Log_{10} reduction	5.2	NA		NA

3.5 γ-Irradiation Inactivation

A pilot study was conducted to empirically determine the appropriate range of γ-irradiation doses that should be examined to calculate the sterility assurance level (SAL). Cobalt-60 was used as a source of γ irradiation for doses of 20, 40, 60, 80, and 100 kGy (2, 4, 6, 8, and 10 million rad). The periods of exposure for these doses were 2.21, 4.42, 6.62, 8.83, and 11.4 h, respectively, at a rate of approximately 0.9×10^6 rad/h. From this study, irradiation doses of ≥20 kGy resulted in complete loss of virus infectivity (data not shown). These results contradict the finding of Reitman and Tribble (1967), in which viable VEE virus was demonstrable at 60 kGy but not at 80 kGy irradiation doses. It is important to note that in the study of Reitman and Tribble (1967), the VEE Trinidad virus was used, and the irradiation exposure was carried out at –70 °C, whereas in our study, the VEE TC-83 virus was exposed at ambient temperature. Nevertheless, the difference between the Reitman and Tribble (1967) study and ours was intriguing and prompted further investigation.

In this study, the observed log reduction ranged from 2.6 to 4.4; therefore, it was not possible to calculate the D_{10} value (the irradiation dose required to achieve a 1 log reduction in the virus titer). Previous studies have suggested D_{10} values of 3.87–10.20 kGy for different species in the Togaviridae family, which includes the VEE virus (Grieb et al., 2005; Jordan and Kempe, 1956; Reitman and Tribble, 1967; Reitman et al., 1970). For managing potential biosecurity risk, the desired SAL is usually set at 10^6, which provides an assurance that there is less than one in a million chance of viable contamination in any one unit. Therefore, two additional studies were conducted to evaluate 1, 5, 10, 15, 20, and 40 kGy using a virus titer ranging from 8×10^6 to 1×10^7 $TCID_{50}$/mL.

The data from two γ-irradiation studies, with results for doses ranging from 1 to 40 kGy (calibrated doses from 1 to 36.7 kGy) on virus infectivity, were combined (Figure 1). As shown in the figure, virus infectivity is reduced linearly with high irradiation doses (as determined by $TCID_{50}$). From these results, it appears that irradiation doses approaching 40 kGy resulted in complete loss of virus infectivity when the virus titer that was used was 1×10^7 $TCID_{50}$/mL.

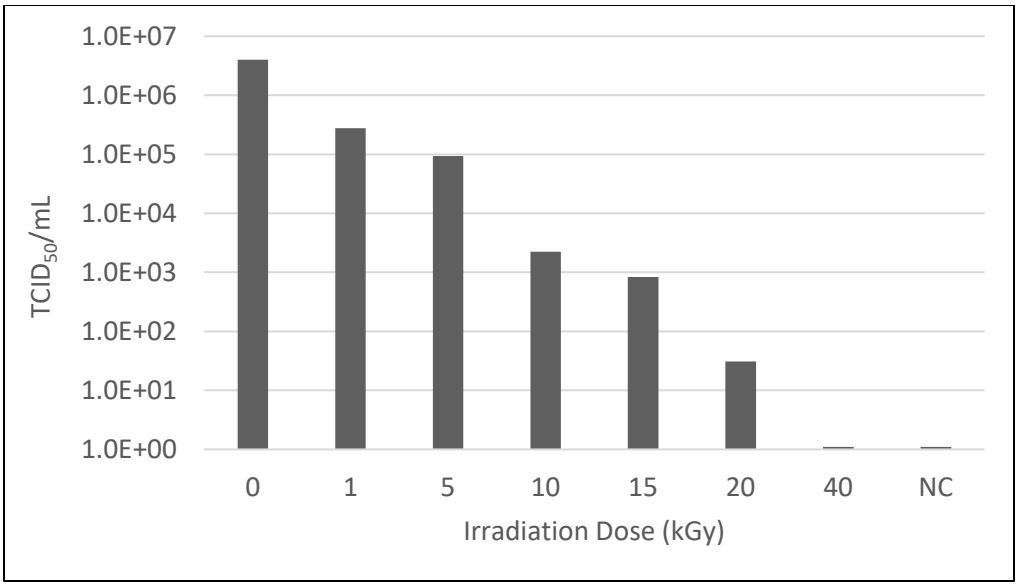

Figure 1. Effect of cobalt-60 irradiation on VEE TC-83 virus infectivity using data from two irradiation studies. NC, negative control.

When analyzed by linear regression (Figure 2), these data showed significant inverse correlation between irradiation dose and virus infectivity (probability [p] = 0.0009; coefficient of determination [R^2] = 0.91).

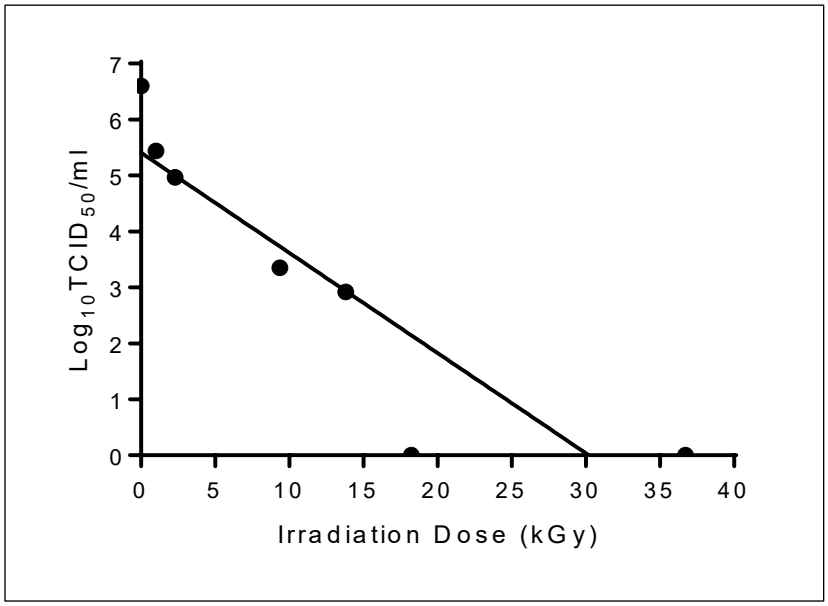

Figure 2. Regression analysis of irradiation dose and log $TCID_{50}$/mL from two irradiation studies (p = 0.0009; R^2 = 0.91; y = –0.1693x + 5.508).

The absolute log-reduction values as determined by the $TCID_{50}$ assays were also analyzed by regression (Figure 3). From this data, we estimated the D_{10} value to be ~5.49 kGy (549,000 rad). The SAL, which is required for 6 log reduction, was estimated to be ~32.92 kGy (3,292,000 rad).

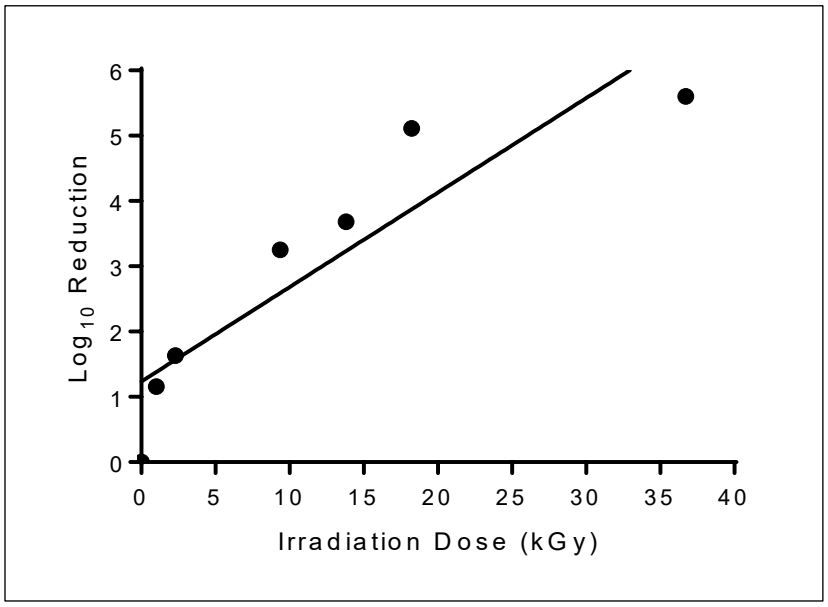

Figure 3. Regression analysis of irradiation dose from two irradiation studies and absolute log reduction ($p = 0.0049$; $R^2 = 0.82$; $y = –0.1447x + 1.236$).

In previous studies, D_{10} values reported for members of the Togaviridae family of viruses ranged from 3.9 to 10.2 kGy. Reitman and Tribble (1967) reported that the D_{10} values for VEE Trinidad varied from 6.04 to 7.15 kGy. According to Grieb et al. (2005), 50 kGy caused a 4.9 log reduction in Sindbis virus, which equates to a D_{10} value of 10.2 kGy. Jordan and Kemp (1956) reported D_{10} values of 5.17 and 5.43 kGy for Western equine encephalitis virus in brain tissues and crude virus, respectively.

In conclusion, based on our results, it appears that a γ-irradiation dose of 30 kGy reduced VEE TC-83 by at least 5 log; therefore, a dose of >30 kGy would be required to achieve the SAL for VEE TC-83. Based on previously published studies by other investigators, we assume that the D_{10} and SAL values for VEE Trinidad are higher than those for VEE TC-83. This assumption will be tested, and the results will be presented in coming reports.

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ACRONYMS AND ABBREVIATIONS

BPL	β-propiolactone
CPE	cytopathic effect
D_{10}	irradiation dose required to achieve a 1 log reduction dose
DPBS	Dulbecco's phosphate-buffered saline
EMEM	Eagle's minimum essential medium
FBS	fetal bovine serum
kGy	kilogray
pen/strep	penicillin and streptomycin
SAL	sterility assurance level
SS+RNA	single-stranded, positive-sense RNA
$TCID_{50}$	tissue-culture infective dose that will result in the infection of 50% of cultures
VEE	Venezuelan equine encephalitis

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